Inroduction:

Cholesterol oxidase (EC 1.1.3.6) is a flavin adenine dinucleotide (FAD) - dependent enzyme that in most cases catalyzes the oxidation of cholesterol (cholest-5-en-3β- ol) using oxygen as an electron acceptor to form cholest-4-en-3-one (CEO) and hydrogen peroxide [1, 2]. During past three decades, cholesterol oxidases (CHO) have been purified from several microorganisms, such as *Brevibacterium* [3] (Gadda et al. 1997), gamma-Proteobacterium [4], *Nocardia* [5] , *Pseudomonas* [6], *Rhodococcus* [7], *Bacillus* [8], *Schizophyllum* and *Streptomyces* [3,9].

However, some cholesterol oxidases from *Burkholderia cepacia* strain ST-200, *Pseudomonas* spp., and *Chromobacterium* sp. strain DS-1oxidize cholesterol to 6β-hydroperoxycholest-4-en-3-one (HCEO) but not the CEO produced by most cholesterol oxidases [10, 11].Since the first report on cholesterol oxidation in *Rhodococcus erythropolis*, microbial cholesterol oxidation has been reported in a number of microorganisms. Stadtman et al.first reported the crude preparation of cholesterol oxidase from a soil *Mycobacterium* [12]. Since then, the enzyme has been isolated and purified from various microorganisms.

There are two forms of cholesterol oxidase, one containing the FAD cofactor non-covalently bound to the enzyme (class I) and another containing the cofactor covalently linked to the enzyme (class II) [13]. These two enzymes have no significant sequence homology. These two forms of enzymes belong to different protein families.

Cholesterol oxidase is widely used for the determination of cholesterol concentrations in serum and other clinical samples [5, 14]. Since the assays employing this enzyme are simple, specific, and highly sensitive compared with the conventional chemical methods, its use has become widespread. In addition, cholesterol oxidase shows insecticidal activity that is a vital part of pest control strategies employing transgenic crops [15, 16]. Moreover, cholesterol oxidase has been used for the optical resolution of no steroidal compounds, allylic alcohols [17, 18], and for the bioconversion of a number of 3β-hydroxysteroids [19, 20].

Objectives:

- To isolate and identify microorganisms from regional oil mill those are able to produce cholesterol oxidase to meet industrial and medicinal needs.
- Optimization of medium for fermentative production of cholesterol oxidase.
- Purification and characterization of cholesterol oxidase.

Work done:

1) Literature survey for isolation of cholesterol oxidase producing microorganisms and methodology for production, purification and characterization of microbial produce cholesterol oxidase.
2) Sample collection.
3) Isolation & screening of cholesterol oxidase producing bacteria.
4) Characterization and partial identification of isolates. (Results of 16sRNA sequences are awaited).
5) Determination of cholesterol oxidase activity.
6) Identification of biotransformation product of cholesterol.
7) Medium optimization and production in laboratory fermenter of cholesterol oxidase.
8) Partial purification of cholesterol oxidase.
9) Kinetic parameters of cholesterol oxidase.
10) Characterization of cholesterol oxidase.

Methodology:

These studies were carried out during the year 2009 to 2012.In this study samples were collected from waste of regional oil mill, agricultural compost and soil. Isolation of CHO producer was carried out as describe by Yazdi M.T. et al [21]. Screening of CHO producing organism were on cholesterol oxidase indicator plates [22].

Characterization of isolates was carried out by studying their morphological, cultural, biochemical and molecular characteristics by standard method. CHO activity was measured by modified method based on the study of Allain et.al [23, 24]. Biotransformation product of cholesterol was identified by thin layer chromatography.

Optimization of medium component and other parameters like temperature and initial pH was carried out by one-factor-at-a-time and orthogonal array method. Laboratory level CHO production carried out in Sartorius stedim, Germany (Biostat A plus, 5 lit. capacity) fermenter.

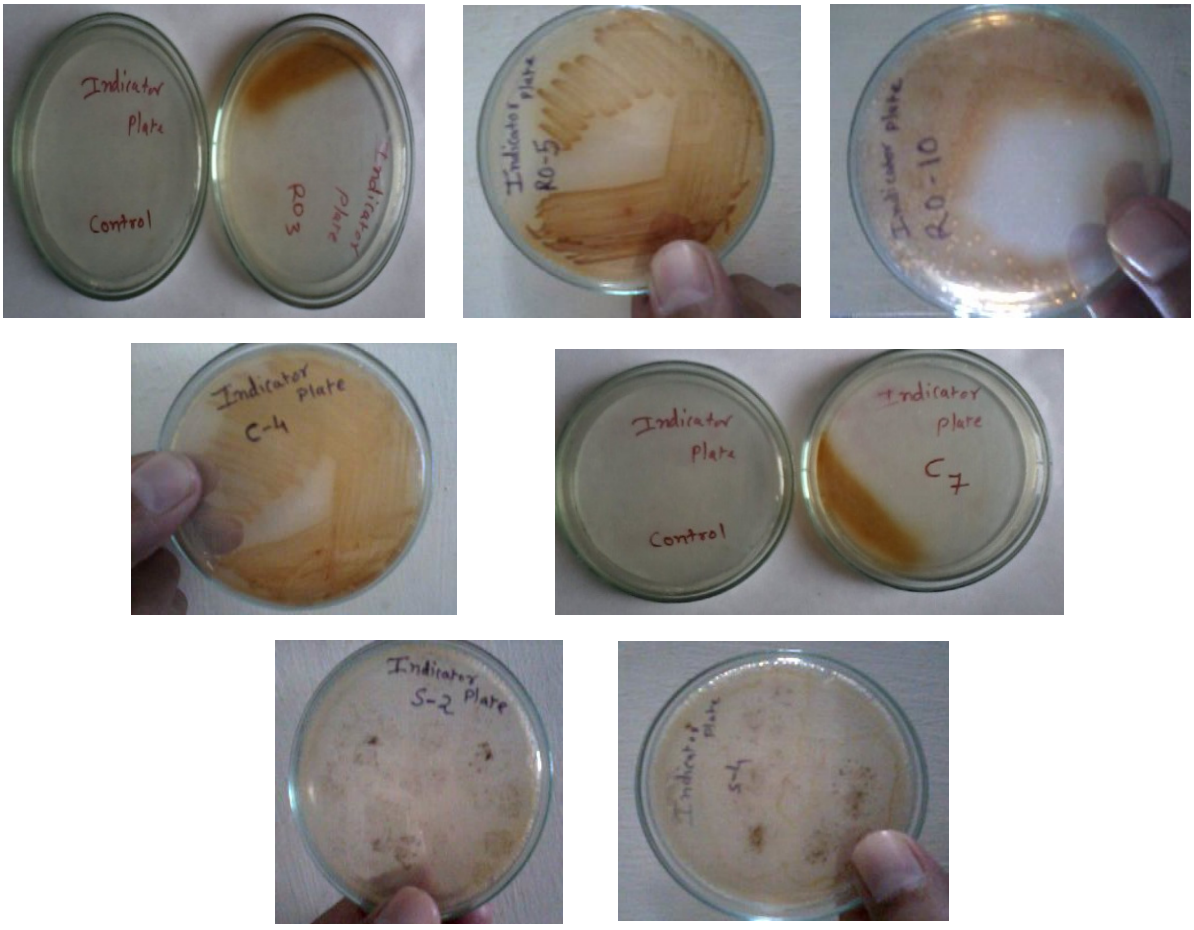
Partial purification was carried out by salt precipitation, dialysis and column chromatography. K_m and V_{max} value of partially purified enzyme was carried out by Lineweaver and burk plot. Characterization of cholesterol oxidase was carried out that includes determination of protein concentration by the method of Lowry et al. [25], determination of molecular weight by SDS-PAGE, determination of pH and temperature profile, determination of absorption maxima, determination of substrate specificity.

Results and Discussion:

Samples were collected from various regional oil industries like Shanidev oil mill, Surat. , Domestic oil manufacturer, Surat. , Ashok industries, Amreli. , Shree savan oil industries, Amreli. , Sahayog Industries, Amreli. , Dhara Vegetable Oil And Foods Company Ltd, Anand. , Agriculture compost and soil etc.

These samples were used for isolation of CHO producing microorganisms by Yazdi M.T. et al [21]. Screening of the various isolates for extracellular CHO production was carried out and biotransformation product of cholesterol was identified by thin layer chromatography. Isolates RO-3, RO-5, RO-10, C-4, C-7, S-2 and S-4 were found as potential extracellular CHO producers.

Figure -1 Growth on Cholesterol oxidase indicator plates



Isolates of waste from regional oil mill RO-3 & RO-10 were identified as *Arthrobacter sp.* and *Streptomyces sp*. RO-11,13 & 15 was identified as *Pseudomonas sp.* Results of 16sRNA for RO-5 is awaited.

Isolates C-7 and C-4 from agriculture compost was identified as *Streptomyces sp.* Two fungal isolates from soil S-2 and S-4 were identified as *Aspergillus sp.* CHO activity from medium B was measured by modified method based on the study of Allain et.al [23, 24].

Figure – 2 Extracellular CHO activitiy of isolates

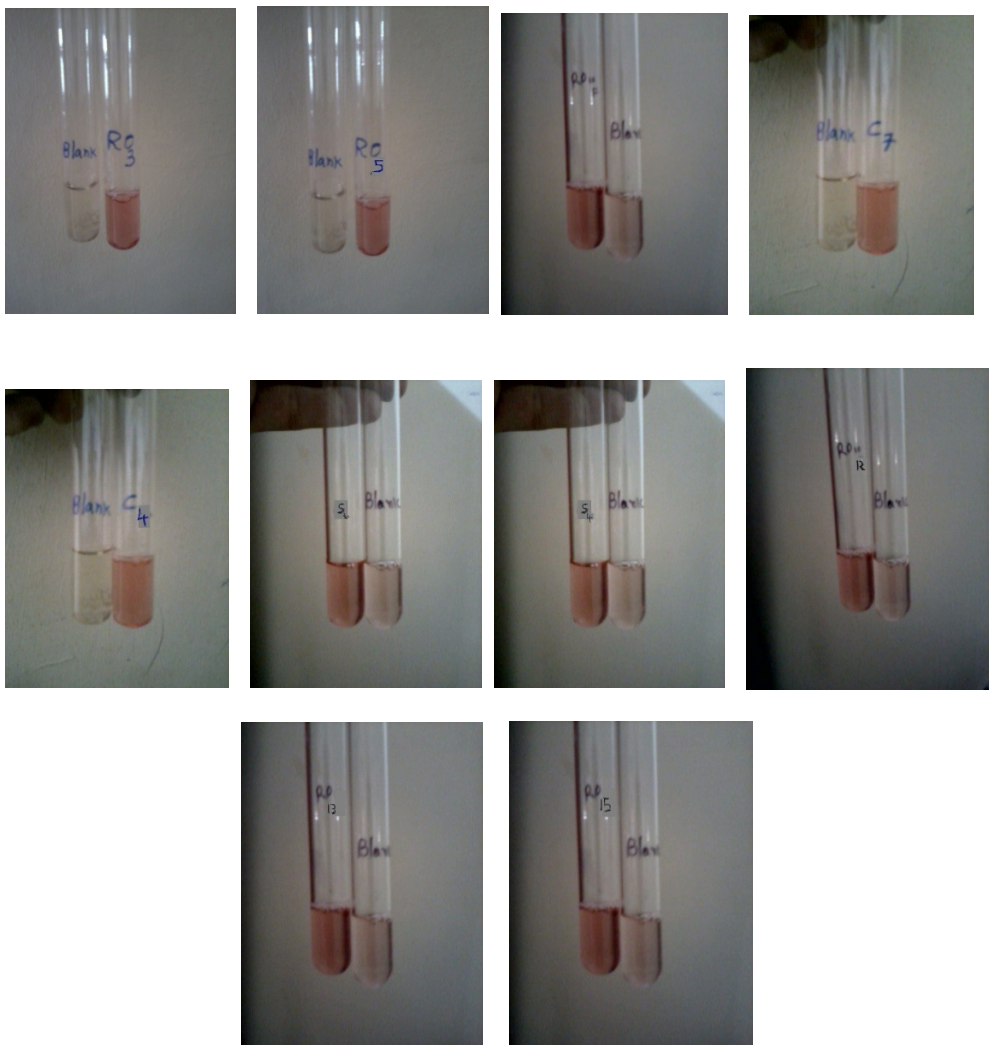
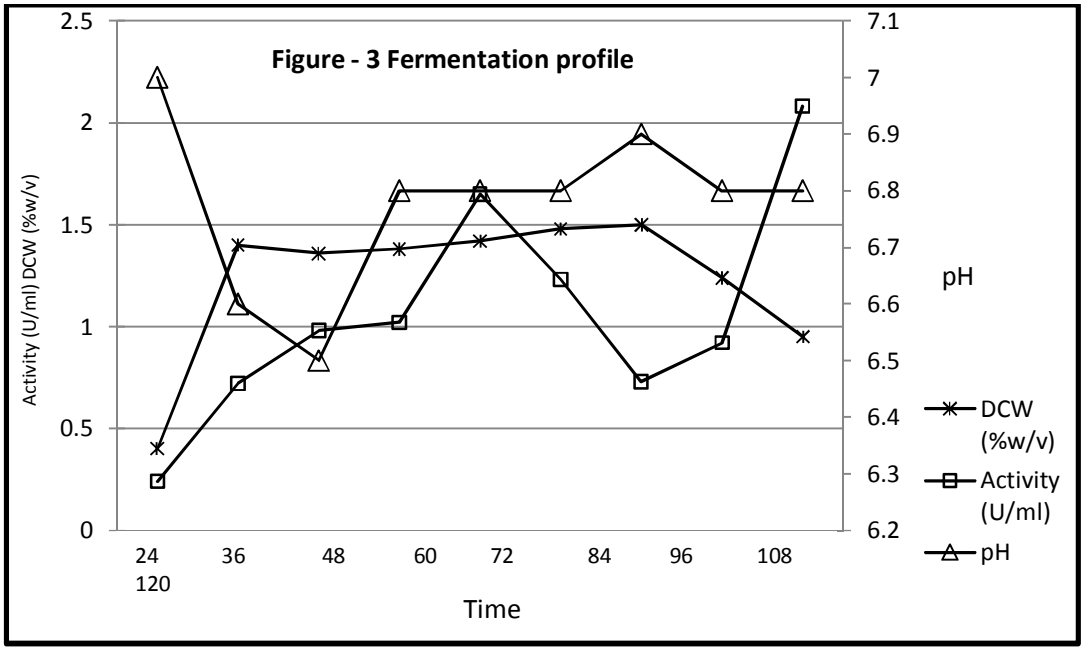
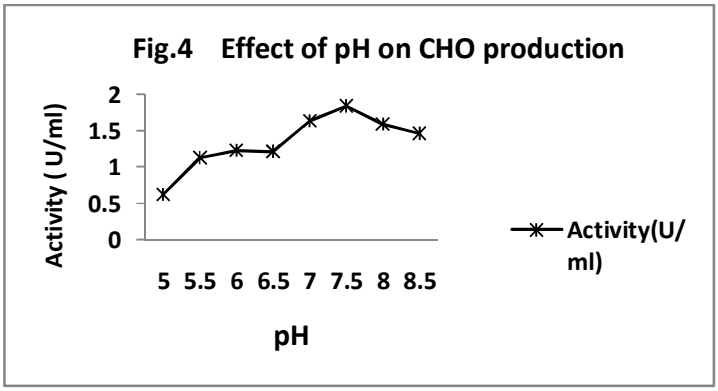


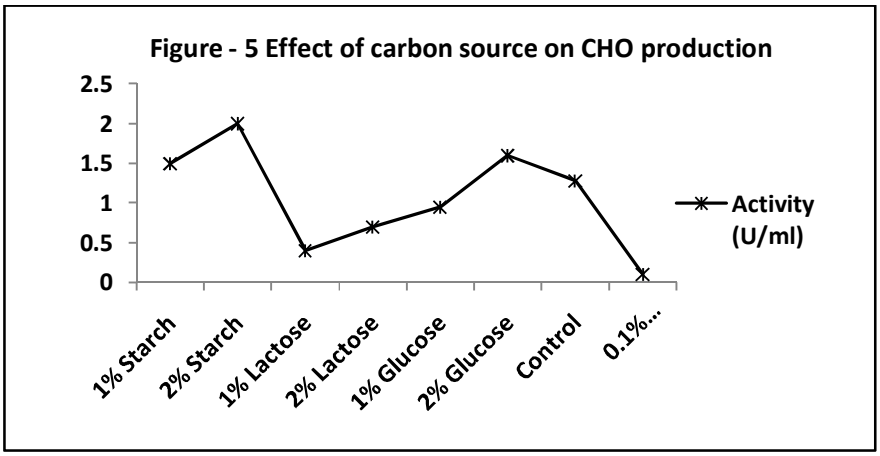
Table-2 Extracellular CHO activity of isolates

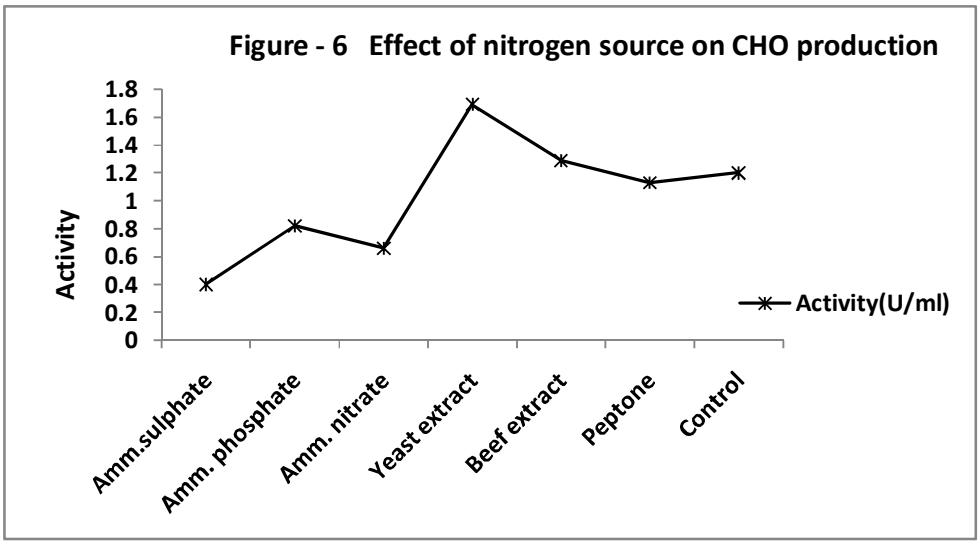
Isolate	Activity (Units/ ml)
RO-3	0.91
RO-5	1.02
RO-10	1.6
RO-11	0.94
RO-13	0.87
RO-15	1.17
C-4	1.1
C-7	1.26
S-2	1.03
S-4	0.97

RO-10 gives highest activity (1.6 unit/ml) among these isolates so; this isolate was selected for further study. Fermentation profile and effect of various initial pH, Carbon source and nitrogen source was studied.









Laboratory level fermentation for CHO production in optimized medium was carried out in laboratory fermenter to obtain CHO in more quantity

for further study. Partial purification of CHO from fermentation broth was carried out by ammonium sulfate precipitation of 60% saturation and precipitates were dissolved in buffer and subjected to dialysis and column chromatography.

Partially purified CHO was use for study of kinetics and determination of optimum pH, temperature, protein concentration, molecular weight, absorption maxima and substrate specificity.

Conclusion:

- *Strptomyces* sp. isolated from the waste of regional oil mill might be considered as a potentially interesting source of extracellular cholesterol oxidase for clinical and commercial purposes.
- The high levels of expression, as well as the ease of purification, encourage us in the belief that this cholesterol oxidase may serve as useful model for bacterial cholesterol oxidases.
- Cholesterol oxidase from Streptomyces sp. is highly stable at high temperatures and in the presence of various organic solvents or detergents. In addition, the enzyme exhibited the low K_m value. In recent years, various biosensors for the determination of cholesterol using the immobilized cholesterol oxidase have been reported [26, 27, 28]. These sensors require a linear and fast response, reproducibility, and stability against variations in pH, temperature, and chemical nature of the microenvironment. Our cholesterol oxidase might improve the usability of these biosensors.
- Thus taking in to account the extracellular production, its efficient recovery, wide pH tolerance, good thermal stability CHO produced by *Streptomyces sp*. should be use for large scale production and cheap and industrially useful carbon and nitrogen source must be investigated.

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